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14. ABSTRACT
Retinoids function upon binding to nuclear retinoid receptors (RARs, RXRs) and have shown promise for the chemoprevention and treatment of prostate cancer. Novel synthetic retinoid-related molecules (RRMs) that function as RARγ/β-selective agonists (MX3350-1, CD2325) or antagonists (MX781) were discovered with strong anticancer activity. These RRMs induce apoptosis independently of RARs. The cellular targets that mediate RRM-anticancer activity are unknown and theie mechanism of action is currently under investigation. The main goal of this project was to identify genes that mediate RRM anticancer activity upon selection of Genetic Suppressor Elements (GSE) that confer resistance to RRM treatment in prostate cancer cells. We have performed several screenings in the presence of toxic amounts of MX781 and MX3350-1. GSEs have been subsequently rescued from surviving cells by PCR amplification using primers specific for the GSE library, followed by DNA sequencing and BLAST homology search for the identification of the corresponding genes. The results of the initial screenings have not been completely reproduced in subsequent experiments and most of the genes tested in functional validation studies have proved to be unnecessary for RRM-induced apoptosis. Our conclusion is that RRMs could activate several independent pathways that converge in apoptosis. Targeting of individual genes may not be sufficient to completely abrogate RRM-mediated cell death.

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Table of Contents

Introduction.

Retinoids are natural and synthetic derivatives of vitamin A that bind and activate the nuclear retinoid receptors (RARs and RXRs) to regulate the expression of target genes. Because of their differentiation and growth inhibitory activities, many efforts have been devoted to develop retinoids as cancer preventive and chemotherapeutic agents. However, very few compounds are clinically useful to humans beyond the treatment of APL and dermatological disorders. This is mostly because of the high levels of toxicity observed at effective retinoid doses, which are likely caused by an RAR/RXR-dependent activity. Therefore, synthetic derivatives have been obtained to selectively activate a subset of retinoid receptors (RAR or RXR agonists; RAR subtype selective agonists) or to inhibit atRA-mediated transactivation (antagonists), which are expected to show lower toxicity. Of particular interest to our program are the adamantyl containing retinoid-related molecules (RRMs) MX3350-1 and MX781. MX3350-1 belongs to the family of RARγ/β-selective agonists represented by CD437, whereas MX781 is an RAR antagonist. These molecules induce apoptosis in a variety of cancer cell lines and MX3350-1 is effective in animal models against solid tumors derived from non-small cell lung carcinomas, whereas MX781 is effective in breast cancer xenograft models. These molecules are strong inducers of apoptosis in prostate carcinoma cells independently of p53 status, and therefore represent promising leads for the discovery of novel retinoid-like molecules as chemopreventive agents in prostate cancer.

Body.

The main goal of our research program is to understand the mechanism of RRM action and to identify genes that mediate their anticancer activity in prostate carcinoma cells. The discovery of genes that are implicated in RRM killing could have a tremendous impact in: i) understanding the mechanism of RRM action in prostate cancer and ii) the discovery of novel therapies that might synergize with currently available retinoids in combination therapies. For this purpose we proposed a Genetic Suppressor Elements (GSE) selection approach as a way to identify genes that mediate RRM action in prostate cancer. GSEs are small fragments of DNA produced by random digestion of a cDNA library that function as antisense DNA (when oriented in antisense direction and are able to decrease expression of a target gene) or as dominant negative fragments of a particular gene product (when expressed in sense orientation). GSEs derived from genes that are required for RRM-induced apoptosis are expected to block cell death in response to RRM treatment. We hypothesized that transfection of a GSE library into PC3 cells should generate cellular clones that would be resistant to RRM killing. GSEs isolated from cells surviving a killing dose of RRM would help us identify genes that mediate RRM function.

The GSE selection approach was first described by Dr. I. Roninson in 1992 using the bacteriophage lambda as a model system (Holzmayer et al., 1992). The technology has evolved tremendously and greatly improved during the following years. Even though this seems as a very powerful genomic approach to achieve our goals, GSE selection is a technologically difficult and risky endeavor, as reflected by the fact that the number of publications and groups reporting on the use of GSE selection has grown very slowly since first described, if we compare to other genomic/proteomic-based strategies. For this reason we sought Dr. Roninson to collaborate in this project and he has provided critical materials to accelerate the work and much needed expertise to problem solving.

A GSE library obtained from MCF-7 cells cloned into the pLmGXC retroviral vector was obtained and amplified as recommended. Briefly, 1 μg DNA was transformed into 4 mls DH5α competent cells using standard conditions. Bacteria were spread on 150 x 150 mm LB/Amp plates and grown overnight. Bacteria were collected and DNA was isolated following standard procedures. This library contains ~10^8 clones and has been optimally used in Dr. Roninson's lab to identify genes required for tumor cell growth (Primiano et al., 2003). We have used the Pantropic Retroviral Expression system

(BD Biosciences, K1063-1) to generate retroviruses carrying a GSE library, which have subsequently been used to infect PC3 cells prior to RRM treatment and GSE selection. Transfection conditions for optimal virus production and infection of PC3 cells were standardized during the first year of the project. A first screening was carried out to obtain PC-3 cells resistant to MX3350-1, which involved two rounds of selection and RRM treatments of 2 weeks. The results of this screening, reported in the second report, were not very encouraging because only one gene (GPX-1) was found twice from 100 individual sequenced colonies. One could expect that true GSE would be represented at much higher numbers in the selected population.

We therefore re-designed the GSE selection strategy and decided to harvest surviving PC3 cells early after a short RRM treatment (24 to 48 hours). Although some of the control pLmGCX-transduced PC3 cells are still alive after 48 hours, we expected that a significantly larger number of cells would survive when infected with viruses carrying the GSE library. We reasoned that if several pathways are activated by RRM that converge in cell death, blocking one pathway would delay the whole process of apoptosis. Furthermore, by collecting cells after a short period of RRM treatment we would avoid loosing weak GSEs and/or GSEs expressed for short periods of time. To obtain meaningful data, early recovery of GSEs will require a high-throughput sequencing of several hundreds of colonies to look for sequence enrichment. Furthermore, following Dr. Roninson's suggestions, we did a high-throughput sequencing of a large number of bacterial colonies instead of a second round of selection. It is possible that some weak GSEs might be lost in this second round.

The production of virus was scaled-up using a CellSTACK culture chamber (Corning) containing 10 stacks with a total of 6,360 cm^2 cell growth area (equivalent to 42 of the 150 mm culture dishes, enough for two to three drug screenings). 320 million GP2-293 cells were incubated with a mixture of Calcium Phosphate/BBS containing 2 mg pLmGCX-GSE library and 1 mg pVSV-G vector. The cell/DNA mixture was added into a poly-D-Lysine treated CellSTACK, placed inside a 3% CO_2 incubator, and incubated for 16 hours. An aliquot of the cell/DNA mix was added into a p100 dish to follow up under the microscope and a control transfection was carried out in parallel with empty pLmGCX vector. After transfection, the medium was carefully removed, cells were washed with PBS, and fresh medium was added; supernatant containing virus was collected each day for the following 5 days, filtered, and quickly frozen at -80 °C. One aliquot for each day stock was reserved for virus titration.

Each GSE selection was performed by seeding $30x10^6$ PC3 cells into 15x150 mm culture dishes (2 M cells per dish). The reason we used a low cell density is because we infected cells three consecutive times 24 h apart. By the time of RRM treatment (24 hours post infection), cells were 80-90% confluent. Cells were treated with 6 µM MX3350-1 or 6 µM MX781 and harvested 48 hours later. Genomic DNA was isolated using a DNeasy Tissue kit (Qiagen) and analyzed by agarose gel electrophoresis. PCR amplification was subsequently performed using 0.5 µg genomic DNA as template and 2.5 U of KlenTaq LA DNA polymerase. As negative control, genomic DNA isolated from non-infected PC3 cells was used. In addition, 0.1 µg of pLmGCX-GSE DNA was used as positive control (Fig 1). The PCR products were cloned into the pCR II-expression vector using a Zero Blunt TOPO PCR cloning kit (Invitrogen). Several hundreds of colonies were obtained from each drug screening.

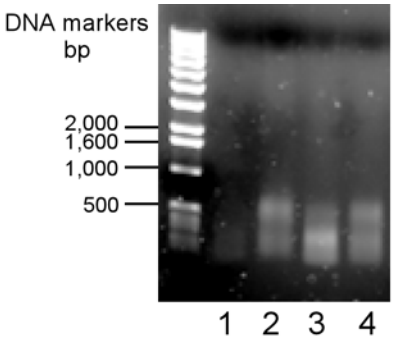

Figure 1. 1% agarose gel analysis of PCR products obtained using genomic DNA from non-infected PC3 cells (lane 1), pLmGCX-GSE library (lane 2), or genomic DNA from GSE-infected PC3 cells treated with MX781 (lane 3) or MX3350-1 (lane 4). Note that the PCR products represent a mixed population of DNA fragments ranging in size between 100 and 500 bp. As expected, DNA isolated from non-infected cells gave no detectable PCR product using primers specific for the creation of the GSE library.

The results of the first screening with MX781 were reported in the previous year report and are summarized in table I.

Table I. Genes represented by two or more GSEs isolated from the **MX781** screening. The number of sequences that appear in sense (s) or antisense (as) orientation is shown.

Gene		Seq (s/as)	Gene Function
	Metabolism		
ARG2	Arginase type 2	2 s	Nitric oxide and polyamine metabolism, mitochondrial protein
CYP27A1	Vit D3 25-hydrolase, Cytochrome P450, family27	2 s	Drug metabolism; synthesis of cholesterol and steroids
	Detoxification		
GPX1	Glutathione Peroxidase 1	1 s, 1 as	H_2O_2 detoxification, antioxidant
PRDX2	Peroxiredoxin 2	2 as	H_2O_2 and alkyl hydroperoxides reduction, antioxidant
	Transcription		
FALZ	Fetal Alzheimer antigen	1 s, 1 as	Transcription regulation
HR	Hairless homolog (mouse)	2 as	Transcriptional corepressor (rats), histone deacetylase interaction
THAP7	Thanatos-associated protein 7	2 as	Transcription repression
ANCO	Ankyrin repeat and SOCS box-containing	1 s, 3 as	Cofactor for p160 nuclear receptor coactivators
	Cell Signaling		
CSK	c-src tyrosine kinase	2 s	Signal transduction, cell cycle progression, transformation
FLNA	Filamin A	4 as	T-cell activation, ERK activation, cytoskeleton reorganization
GEF	Rho guanine nucleotide exchange factor	8 s	Rho signaling regulation
ICMT	Isoprenylcysteine carboxyl methyltransferase	1 s, 1 as	Post-transcriptional protein modification, protein targeting to cell membrane, transformation, apoptosis
NPIP	Nuclear pore complex interacting protein	2 as	Signal transduction, cell cycle progression, transformation
SPRY2	Sprouty-related 2 (Drosophila)	3 as	Regulator of EGFR and MAPK signaling,

			cell proliferation inhibition
STMN1	Stathmin 1	2 as	Integrates regulatory signals, promotes microtubule filament depolymerrization
UBL7	Ubiquitin-like 7	2 s	Signal transduction, cell proliferation, apoptosis
WDR 34	WD repeat domain 34	2 as	Signal transduction, cell cycle progression, apoptosis
YWHAZ	Tyrosine 3-monoxygenase/tryptophan monoxygenase activation protein	2 as	14-3-3 Family member, phosphoserine-protein interaction
ADAM17	ADAM metallopeptidase	3 as	Cell-cell and cell-matrix interactions, activation of Notch signaling pathway
PQBP1	Polyglutamine binding protein	3 s	Role in mitochondrial stress
MRLC2	Myosin regulatory light chain	2s	Myosin II filament assembly
	Protein processing		
TUBB2C	Tubulin beta 2C	2 as	Chaperon, unfolded protein binding, microtubule-based motility
PPIH	Cyclophilin H	2 as	Chaperon with function in splicesosome, peptydil/prolyl isomerase
	Cellular transport		
DCTN2	Dynactin 2	2 s	ER-to-Golgi transport, organelle movement, spindle formation, chromosome movement
EXO7	Exocyst complex componet 7	3 s	Exocytosis, tubulin polymerization
NUP188	Nucleoporin 188 kDa	3 as	Porin activity
	Development		
DHX35	DEAH box polypeptide 35	3 as	Embriogenesis, cell growth and division, ATP depemdent helicase
NOMO2	NodaL modulator 2	2 s	Development
WHSC1	Wolf-Hirchhom syndrome	2 s	Early development, methyltransferase activity

As we discussed in our previous report, none of the genes depicted in Table I had an obvious role on apoptosis, although some are protein kinases and important cell signaling molecules that might be required for MX781-induced cell death. GEF12 is the most frequently found gene, which is a Rho guanine nucleotide exchange factor. Some GEF family members have been involved in apoptosis, including GEF-H1 and p115-RhoGEF. The fact that this sequence has been found with relative high frequency in this screening encouraged further consideration. Since our preliminary results show that MX781 induce apoptosis via mitochondrial damage and oxidative stress, GSEs corresponding to proteins with a role in mitochondrial function and the redox state of the cell, such as ARG2 (arginase type 2), GPX-1 (glutathione peroxidase 1), PRDX2 (peroxiredoxine 2), are of particular significance for subsequent functional studies. Interestingly, GPX1 was the only gene that was found twice in our original screening with MX3350-1. This RRM also induces oxidative stress in prostate carcinoma cells (data not shown) and therefore may share with MX781 this oxidative pathway. Although GPX-1 is an antioxidant enzyme that prevents oxidative stress-induced apoptosis, it might be possible that drug treatment converts GPX-1 into a pro-apoptotic molecule in prostate carcinoma cells. This warranted future validation of GPX-1.

Because the number of repeated sequences was unexpectedly low, we decided to carry out a second screening with MX781 and to analyze the GSEs rescued from the previous MX3350-1-screening. As before, genomic DNA was isolated from PC-3 infected cells that were treated with 6 µM MX781 for 48 h. This time we decided to sequence DNA directly from the bacterial colonies, without isolating DNA first for restriction analysis. Of the ~600 colonies sequenced, only ~50% came back with readable sequences of ≥100 nt. We performed BLAST analysis of these 326 sequences and found no repeated sequences. Moreover, the sequences found in this second screening were not related to the genes found in our first screening described in Table I. This was clearly unexpected and inexplicable, because both sets of colonies came from the same cloning. Only the GPX-1 sequence was found once in this second GSE analysis.

Although we gain time by sequencing directly form the bacterial colony, it is clear that the results are not as encouraging as those obtained in the more labor-intensive approach of isolating DNA and analyzing individual colonies by enzyme restriction prior to sequencing. We therefore used this second strategy to analyze GSEs rescued form the second MX3350-1 screening. We sequenced 768 insert containing DNAs out of ~1300 bacterial isolates. The genes found repeated at least twice are shown in Table II.

Table II. GSEs producing resistance to **MX3350-1**

Gene		Seq (s/as)	Gene Function
	Detoxification		
GPX1	Glutathione Peroxidase 1	4 as	H_2O_2 detoxification, antioxidant
	Transcription		
HR	Hairless homolog (mouse)	4 s, 1 as	Transcriptional corepressor (rats), histone deacetylase interaction
ANCO	Ankyrin repeat and SOCS box-containing	2 as	Cofactor for p160 nuclear receptor coactivators
	Cell Signaling		
GEF	Rho guanine nucleotide exchange factor	2 s	Rho signaling regulation
JIP-3	JNK-interacting protein	1 s, 4 as	JNK scaffold
MAPKAPK5	MAP kinase-activated protein kinase 5	2 s, 1 as	Regulator of MAPK signaling
TNFRSF1B	TNF receptor superfamily member 1B	3 as	TNF signaling
WDR 34	WD repeat domain 34	5 as	Signal transduction, cell cycle progression, apoptosis
	Cellular transport		
NPIP	Nuclear pore complex interacting protein	5 as	Signal transduction, cell cycle progression, transformation
	Others		
DDX35	DEAD/H box polypeptide 35	1 s, 5 as	
eEF1G	eukaryotic translation elongation factor 1 gamma	1 s	Function in protein translation
18S rRNA			Ribosome structure
RPS23	Ribosomal protein 23	2 as	Ribosome structure

RPS2	Ribosomal protein 2	1 as	Ribosome structure
TMEPAI	Transmembrane prostate androgen induced gene	2 s	

From this screening, several genes are worth to mention. We found again GPX-1 repeated, as we did in the original screening as well as in the MX781 screening. GPX-1 is clearly a candidate for validation. From independent studies in our lab, we know that MX3350-1 and other related agonists induce apoptosis via JNK activation (Ortiz et al., 2001; Piedrafita and Ortiz, 2006). Following studies have demonstrated that CD437-like RRMs (including MX3350-1) activate JNK/p38 and induce apoptosis via ribotoxic stress (Iordanov et al., 1997) (our unpublished observations). Therefore, it was expected to find GSEs that inhibit genes of the JNK pathway, such as the JNK scaffold protein JIP-3 and MAPKAPK5. In addition, it is interesting that certain ribosomal proteins and even rRNA were found in the GSE screen. These genes may have a function on the RRM-induced ribotoxic stress response. Another gene on the same trend is eEFiG, an eukaryotic elongation factor with a role in protein translation (ribotoxic stress is mostly caused by protein synthesis inhibitors that cause ribosome damage, such as anisomycin).

We selected the following genes for functional validation: GPX-1, PRDX2, CSK, GEF12, JIP-3, MAPKAPK5, EEF1G, RPS2 and RPS23. Our first approach was to use individual GSEs. From the TOPO-PCR clones, we digested miniprep DNA containing the before mentioned GSEs with Bgl II, purified fragments by agarose gel, and cloned into Bgl II-linearized pLmGCX vector. These were transfected into GP2-293 cells seeded in p100 dishes for virus production. As we did during the screening, virus was collected every 24 h for 5 days, filtered and frozen at -80°C until use. PC-3 cells were seeded in p100 dishes (0.5 M per dish) and infected with each individual GSE-carrying viral preparation. Twenty-four hours after the third infection, cells were treated with 6 µM MX3350-1, MX781, or DMSO, as control. Cells were also infected with virus carrying empty pLmGCX vector. Cells were observed daily over the following days for the appearance of apoptosis. 24 h following RRM treatment, control untreated cells were healthy with no obvious signs of apoptosis. However, RRM-treated cells infected with empty pLmGCX vector showed clear signs of apoptosis after 24 h of treatment (cells were rounded and beginning to de-attach). Unexpectedly, all RRM-treated cells showed clear signs of apoptosis even when infected with GSE-carrying virus.

We speculate that individual GSEs are very weak and may not exert the same effect as when transfected in a pool library. To further evaluate the potential role of candidate genes in RRM-induced apoptosis, we decided to use a silencing RNA strategy. Individual siRNAs are available from Dharmacon to target almost every single gene in the human genome. We obtained the On-TARGET Smart pool reagents to target the following genes (although we also planned to target PRDX2 and RPS2, no siRNA reagents are currently available): GPX-1 (L-008982-00), GEF-12 (L-008480-00), EEF1G (L-017546-01), RPS23 (L-011154-01), and MAPKAPK5 (L-005015-00).

These reagents are guaranteed to inhibit expression of target genes by at least 80% after 24-48 hours following transfection. We therefore transfected PC-3 cells with the individual siRNAs as well as a GAPDH siRNA as control. 24, 48, and 72 h after transfection we isolated RNA and analyzed the expression of the individual genes by qRT-PCR. All genes were silenced as expected (data not shown). We then evaluated the induction of apoptosis by RRMs in siRNA-transfected cells. As before, PC-3 cells were transfected with the individual siRNA reagents. 48 h after transfection (optimal silencing time), cells were trypsinized and distributed into 3 wells of a 6 well plate. Cells were then treated with DMSO, or 6 µM of MX781, MX3350-1. 24 hours after treatment, cells were harvested and analyzed for DEVDase activity as a measure of apoptosis. Figure 2 shows the result of the assay.

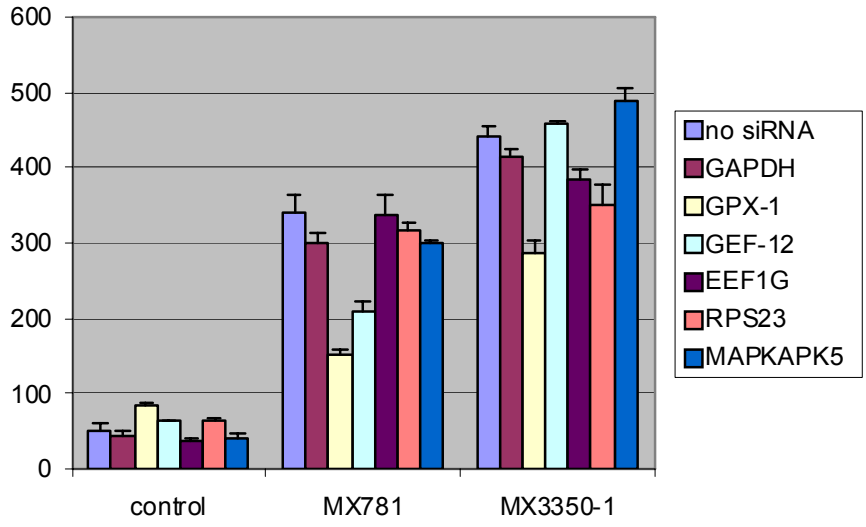

Figure 2. Induction of apoptosis (DEVDase activity) in cells lacking candidate GSEs. PC-3 cells were transfected with the indicated siRNAs. Silencing efficiency was monitored by qRT-PCR (not shown). Following transfection, cells were treated with DMSO (control), 6 µM MX781, or 6 µM MX3350-1 for 24 h, when cells extracts were prepared and analyzed for DEVDase activity.

As depicted in figure 2, silencing GPX-1 partially prevented MX781-induced apoptosis. Similarly, partial block of MX3350-1 was seen, although the effect was significantly lower. The guanine nucleotide exchange factor GEF-12 also had a partial effect on MX781-mediated cell death. When cells were stimulated with the RAR agonist MX3350-1, deficiency of RPS23 or eEF1G partially blocked the activation of caspases.

In summary: we selected a few genes that were most represented in the MX781 and MX3350-1 screenings for functional validation. Validation with individual GSEs did not result in RRM-resistant cells. However, when genes were silenced by modern siRNA strategies, several genes elicited a partial block of RRM-induced apoptosis (GPX-1 and GEF on MX781; GPX-1, eEF1G and RPs23 on MX3350-1)

Research accomplishments.

- GSE rescue and DNA analysis of MX3350-1-resistant cells.
- Identification of genes involved in ribosome structure and protein synthesis: EEF1G, 18S rRNA, RPS2 and RPS23
- Identification of genes of the JNK/p38 pathway, JIP-3 and MAPKAPK5, which is necessary for RRM-induced apoptosis.
- Other genes also found in the screening with MX781: GPX-1 and GEF.
- Validation studies with individual GSEs: cells were not resistant to MX781 or MX3350-1 when cells were infected with GSE-carrying virus.
- Validation with siRNA. Silencing of GPX-1 or GEF-12 partially blocked MX781-induced apoptosis.
- Cells lacking GPX-1, EEF1G, or RPS23, were partially resistant to MX3350-1.

Reportable Outcomes.

No reportable outcomes are available at this time. Additional experiments will be performed independently of this proposal to further understand the role of GPX-1, GEF12, EEF1G, and RPS23 on RRM-induced apoptosis. At least one manuscript will be published to describe these results.

Conclusions.

Screenings have been repeated for both RRMs in order to confirm the results of the initial screening. Although genes were not repeated at a high rate and the results of two independent experiments were not very reproducible as one would have desired, we decided to go ahead with the functional validation of selected genes that were good candidates because of their known function and what it is known on RRM mechanism. Using siRNA techniques, we were able to validate some of the identified GSEs.

Assuming the risk of this project, we have learnt that RRMs can induce apoptosis via many different pathways and that blocking one pathway (GSE, siRNA, others) many not be sufficient to completely abrogate RRM-induced apoptosis. This GSE technology, although difficult and risky, has proven worthy of use. Thus, additional screenings with other libraries may provide additional information to further understand the mechanism of RRM action in prostate cancer.

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